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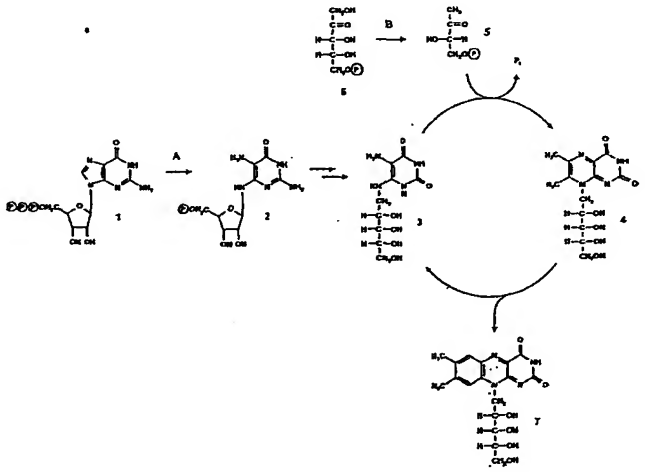
(54) Title: SCREENING FOR INHIBITORS OF LUMAZINE SYNTHASE



(57) Abstract: A method is described for screening for the presence or absence of inhibition of 6,7-dimethyl-8-ribityllumazine synthase activity comprising the following steps : (a) preparing a first aqueous mixture containing a protein having a plant-type 6,7-dimethyl-8-ribityllumazine synthase sequence, 5-amino-6-ribitylamino-2,4(1H,3H)pyrimidine-dione and 3,4-dihydroxy-2-butanone 4-phosphate; (b) reacting said first mixture during a predetermined period of time at a predetermined temperature and subsequently detecting the level of 6,7-dimethyl-8-ribityllumazine; (c) preparing a second aqueous mixture by including in said first mixture a predetermined amount of a chemical test sample; (d) reacting said second mixture during the predetermined period of time at the predetermined temperature and subsequently detecting the level of 6,7-dimethyl-8-ribityllumazine; (e) determining the presence of inhibition of 6,7-dimethyl-8-ribityllumazine synthase by observation of whether the level detected in step (d) is lower than the level detected in step (b).

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INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 38986 A (NOVARTIS ERFIND VERWALT GMBH ;NOVARTIS AG (CH); GUYER CHARLES DAVI) 5 August 1999 (1999-08-05) the whole document --- -/--	1-12

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 February 2001	02/03/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Gundlach, B

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Form PCT/ISA/210 (second sheet) (July 1992)

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JORDAN DOUGLAS B ET AL: "Plant riboflavin biosynthesis. Cloning, chloroplast localization, expression, purification, and partial characterization of spinach lumazine synthase." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 274, no. 31, 30 July 1999 (1999-07-30), pages 22114-22121, XP002153695 ISSN: 0021-9258 abstract	11,12
X	-& DATABASE EMBL - TREMBL 'Online! ID/ACC: 080575, 1 November 1998 (1998-11-01) ROUNSLEY, S D ET AL.: XP002160740 abstract	11,12
P,X	WO 00 44727 A (KIS KLAUS ;BACHER ADELBERT (DE); KUGELBREY KARL B (DE); MIHALIC JE) 3 August 2000 (2000-08-03) the whole document	1-12
P,X	WO 00 40744 A (HERZ STEFAN ;BACHER ADELBERT (DE)) 13 July 2000 (2000-07-13) the whole document	11,12
P,X	EP 1 010 760 A (DU PONT) 21 June 2000 (2000-06-21) example 10	1-12
E	US 6 146 866 A (BACOT KAREN ONLEY ET AL) 14 November 2000 (2000-11-14) the whole document	1-12

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 13,14

Present claims 13, and 14 relate to compounds or a method involving such compounds defined by reference to a desirable characteristic or property, namely being identifiable by the screening methods of claims 1-10.

The claims cover all compounds having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for no such compounds. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the compounds by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the screening methods per se.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. ional Application No
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9938986 A	05-08-1999	NONE	
WO 0044727 A	03-08-2000	DE 19903736 A	03-08-2000
		AU 2293700 A	18-08-2000
WO 0040744 A	13-07-2000	DE 19942174 A	21-06-2000
		AU 1979500 A	24-07-2000
EP 1010760 A	21-06-2000	JP 2000152791 A	06-06-2000
US 6146866 A	14-11-2000	NONE	

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(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/18233 A2

(54) Title: METHOD FOR SCREENING FOR INHIBITORS OF THE BIOSYNTHESIS OF RIBOFLAVIN

(57) Abstract: A method is described for screening for the presence or absence of inhibition of 6,7-dimethyl-8-ribityllumazine synthase activity comprising the following steps : (a) preparing a first aqueous mixture containing a protein having a plant-type 6,7-dimethyl-8-ribityllumazine synthase sequence, 5-amino-6-ribitylamino-2,4(1H,3H)pyrimidine-dione and 3,4-dihydroxy-2-butanone 4-phosphate; (b) reacting said first mixture during a predetermined period of time at a predetermined temperature and subsequently detecting the level of 6,7-dimethyl-8-ribityllumazine; (c) preparing a second aqueous mixture by including in said first mixture a predetermined amount of a chemical test sample; (d) reacting said second mixture during the predetermined period of time at the predetermined temperature and subsequently detecting the level of 6,7-dimethyl-8-ribityllumazine; (e) determining the presence of inhibition of 6,7-dimethyl-8-ribityllumazine synthase by observation of whether the level detected in step (d) is lower than the level detected in step (b).

Method for screening for inhibitors of the biosynth sis of riboflavin

The invention relates to a method for screening for inhibitors of the biosynthesis of riboflavin. It further relates to plant-type enzymes for said method as well as DNA coding for said enzymes. Finally it relates to a method of inhibiting the biosynthesis of riboflavin in plants as well as chemical compounds exhibiting such inhibition.

A promising new approach for finding novel types of herbicides consists in screening libraries of chemical test samples for compounds that inhibit an enzyme in a biochemical pathway that is essential for plants but not for humans or animals. A most promising biosythetic pathway of this type is the pathway of riboflavin biosynthesis. All cellular organisms require riboflavin as an indispensible component of numerous redox enzymes many of which are crucial for the metabolism. All plants generate riboflavin biosynthetically, whereas all animals require a nutritional source of riboflavin. Therefore, an inhibitor for an enzyme in the biosynthesis of riboflavin in plants would not interfere with the metabolism of animals. Furthermore, the absolute amount of riboflavin for cellular activity is low. Therefore, only small amounts of the enzymes for riboflavin biosynthesis are found in cells. This in turm means that only small amounts of an inhibitor for such an enzyme would be-required.

The biosynthetic pathway of riboflavin (Fig. 1) has been studied in considerable detail in bacteria and yeast. The biosynthetic formation of one molecule of riboflavin (7) requires one molecule of GTP and two molecules of ribulose 5-phosphate. GTP (1) is initially converted to the committed product, 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinedione (3) by a sequence of

deamination, side chain reduction, and dephosphorylation. The compound (3) is converted to 6,7-dimethyl-8-ribityllumazine (4) by condensation with 3,4-dihydroxy-2-butanone 4-phosphate (5) wich is obtained enzymatically from ribulose 5-phosphate (6).

It is an object of the invention to provide a method for screening for inhibitors for an enzyme in the plant biosythesis of riboflavin, or for screening for an enzyme that is resistant to a specific inhibitor. It is a further object of the invention to provide a protein having an enzyme activity, useful in such screening method, as well as DNA coding for said protein.

It is further an object of the invention to provide inhibitors as well as a method for inhibiting an enzyme in the biosythesis of riboflavin.

We have discovered that the genomes of plants, specifically *Arabidopsis thaliana*, comprise a gene that codes for a protein that comprises a leader sequence and a sequence exhibiting 6,7-dimethyl-8-ribityllumazine synthase activity. We have expressed this enzyme and found that it is useful for screening a chemical library for inhibitors, with or without the leader sequence.

Specifically we have provided a method for screening for the presence or absence of inhibition of 6,7-dimethyl-8-ribityl-lumazine synthase activity comprising the following steps:

(a) preparing a first aqueous mixture containing a protein having a plant 6,7-dimethyl-8-ribityllumazine synthase sequence, 5-amino-6-ribitylamino-2,4(1H,3H)pyrimidine dione and 3,4-diyhydroxy-2-butanone 4-phosphate,

(b) reacting said first mixture during a predetermined period of time at a predetermined temperature and subsequently detecting the level of 6,7-dimethyl-8-ribityllumazine,

(c) preparing a second aqueous mixture by including in said first mixture a predetermined amount of a chemical test sample,

(d) reacting said second mixture during the predetermined period of time at the predetermined temperature and subsequently detecting the 6,7-dimethyl-8-ribityllumazine,

(e) determining the presence of inhibition of 6,7-dimethyl-8-ribityllumazine synthase by observation of whether the level detected in step (d) is lower that the level detected in step (b).

Further we have provided a method for screening for the presence or absence of resistance to inhibition of 6,7-dimethyl-8-ribityllumazine sythase activity comprising the following steps:

(a) preparing a first aqueous mixture containing a protein having a mutated plant-type 6,7-dimethyl-8-ribityllumazine synthase sequence, 5-amino-6-ribitylamino-2,4(1H,3H)pyrimidinedione and 3,4-dihydroxy-2-butanone 4-phosphate,

(b) reacting said first mixture during a predetermined period of time at a predetermined temperature and subsequently detecting the level of 6,7-dimethyl-8-ribityllumazine,

(c) preparing a second aqueous mixture by including in said first mixture a predetermined amount of a specific inhibitor for 6,7-dimethyl-8-ribityllumazine synthase activity,

(d) reacting said second mixture during the predetermined period of time at the predetermined temperature and subsequently detecting the level of 6,7-dimethyl-8-ribityllumazine,

(e) determining the presence of resistance to inhibition of 6,7-dimethyl-8-ribityllumazine synthase by observation of whether the level detected in step (d) is similar to the level detected in step (b).

Based on the screening method we have thus provided a general solution of the problem of providing inhibitors as well as a method of inhibition for riboflavin biosynthesis in plants.

The invention will now be described in detail.

Identification of the lumazine synthase gene of *Arabidopsis thaliana*
The known amino acid sequence of the ribE protein of *E. coli* (Mörtl et al.) was used to search the DNA sequence data base of the Institute for Genomic Research (Rockville, USA) (accession number, AC004005). Significant similarity was found with a segment of BAC clone F6E13 from *Arabidopsis* chromosome II (Fig.1). The exons of the sequence had been predicted by the computer program xgrail and the predicted protein had been incorrectly assigned as riboflavin synthase.

Sequence comparison showed that the *E. coli* ribE protein was similar over its entire length to the sequence predicted by the *Arabidopsis* gene F6E13.18. However, the putative lumazine synthase gene of *Arabidopsis* also specifies an N-terminal peptide sequence with a high content of serine and threonine (about 67 amino acids) which is devoid of similarity to any sequence in the database and has no equivalent in the *E. coli* protein.

Cloning, expression and purification of lumazine synthase from *Arabidopsis thaliana*

Originator for the construction of an expression vector is cDNA. The *ribE* gene is amplified by PCR with specific primers and cDNA from the corresponding plant as template. Alternatively the cDNA may originate from an existing EST-clone. The RibE protein of *A. thaliana* includes a signal sequence of about 67 amino acids which was found not to be essential for enzyme activity.

The amplified DNA fragment is modified by two consecutive PCR amplifications with modifying primers. In the first PCR reaction a ribosomal binding site preceding the start codon at an optimal distance is introduced at the 5´-end. A recognition site for a restriction enzyme, for example BamHI, SalI or PstI is introduced at the 3´-end. The preferred recognition site is BamHI. In the second PCR reaction the product of the first is used as template. At the 5´-end a recognition site for the restriction enzyme EcoRI preceding the ribosomal binding site is introduced with a modifying primer. The amplified DNA fragment is inserted into a vector capable of autonomous replication in the host microorganism to give a recombinant plasmid containing said DNA. The recombinant plasmid is used to transform the host microorganism, for example *Escherichia coli* or *Bacillus subtilis*. The preferred host is *E. coli*. The expression of plant protein may be poor in the host organism. To enhance the expression level and/or to simplify the purification of the protein the recombinant plasmid may include a gene or a part of a gene without a stop codon preceding the *ribE* gene in the same reading frame. A preferred gene for this purpose is the *malE* gene from *E. coli*. The expression of such a recombinant DNA generates a fusion protein between the maltose binding protein (MBP) from *E. coli* and the plant RibE protein. Various constructs are shown in Fig. 3. Fig. 3A shows a construct with putative signal sequence S, Fig. 3B shows a construct without the putative signal sequence. Fig. 3C shows a construct with maltose binding protein and without putative signal sequence.

The strains harbouring the expression vectors can be cultivated in conventional culture media at 15 to 40°C. The preferred temperature is 37°C. The *E. coli* strains are induced with 0.5 to 2 mM isopropyl-β-D-thiogalactosid at an optical density from 0.5 to 0.8. The cells are incubated between 2 and 12 h, preferably 5 h. The cells are lysed with lysozyme and/or disrupted with a sonifier. The crude extract with MBP-ribE fusion protein is purified by affinity chromatography with an amylose resin. A protein is obtained which has the proper folding

structure for exhibiting the desired enzyme activity.

Screening for the presence or absence of inhibition of 6,7-dimethyl-8-ribityllumazine synthase

Lumazine synthase catalyzes the formation of 6,7-dimethyl-8-ribityllumazine by condensation of 5-amino-6-ribitylamino-2,4(1H,3H)pyrimidinedione with 3,4-dihydroxy-2-butanon 4-phosphate (Neuberger et al., 1986; Volk & Bacher, 1988). The enzyme requires no cofactors and shows full catalytic activity in the presence of a chelator such as EDTA. 3,4-dihydroxy-2-butanon 4-phosphate is prepared enzymatically from ribulose 5-phosphate by the action of 3,4-dihydroxy-2-butanon 4-phosphate synthase (Richter et al., 1992). The assay can be started by adding one of the needed substances to a mixture of the others. Preferably 5-amino-6-ribitylamino-2,4(1H,3H)pyrimidinedione is added to a solution of 3,4-dihydroxy-2-butanon 4-phosphate and enzyme in a buffer at pH 6.5 to 8.0, preferably 7.0. The reaction mixture can be incubated for 1 to 60 min at 10 to 40°C. Preferably it is incubated for 5 min at 37°C. The assay can be stopped by denaturing the enzyme with trichloroacetic acid, acetone or sodium dodecylsulfate. The preferred denaturing is with trichloroacetic acid. The assay is carried out with otherwise identical mixtures with and without test sample of a possible inhibitor. The enzyme product 6,7-dimethyl-8-ribityllumazine can be detected directly without derivatization, preferably photometrically, preferentially after purification by HPLC. The lumazine can be identified by absorbance at 410 nm. The extinction coefficient is 10300 $M^{-1}cm^{-1}$. The product can also be monitored fluorometrically at an absorbance wavelength at 408 nm and an emision wavelength at 487 nm. 6,7-Dimethyl-8-ribityllumazine can also be synthesized without enzymatic catalysis (Bacher et al., 1996). For an exact determination of the enzymatic activity it is therefore necessary to subtract the amount of lumazine formed non-catalytically from the entire amount of produced lumazine.

The isolated DNA codes specifically for a protein with a plant-type sequence of 6,7-dimethyl-8-ribityllumazine synthase whereby it may have either a single open reading frame for said protein or additionally at least one further open reading frame coding for another enzyme of the flavin pathyways.

The term "plant-type sequence" means in one sense a sequence as it occurs in a plant (with or without leader sequence). In a broader and more adequate sense, it means a sequence of a sequence space which is established by using a specific plant 6,7-dimethyl-8-ribityllumazine synthase (with or without leader sequence) as a reference sequence, producing an alignment of said reference sequence with at least one other plant 6,7-dimethyl-8-ribityllumazine synthase sequence and obtaining for each position in said alignment a set of equivalent amino acids from the variability at this position. Any sequence contained in this sequence space has a very high likelihood of being functional for the intended enzyme activity and being highly homologous to plant enzymes.

It is surprising that the isolated proteins could be obtained in a form (e.g. folding form) which is functional for the intended enzyme activity. This functional competence defines a subset of substances of the much broader set of substances which all have the same sequence.

For the screening process, a specific plant enzyme may be used selected in accordance with the plant(s) targeted. It is also possible to construct a plant-type enzyme sequence (with or without leader sequence) which is on average closest to each of a subset of plant enzyme sequences of a set of plants targeted.

Example 1: Construction of an expression clone

The putative *ribE* gene (gene F6E13.18) of E. coli was amplified by PCR using a cDNA library (Minet et al.) as template
First PCR:

The reaction mixtures contained 10 pmol primer 5´-GGAGAAATTAACCA TGAAGT-CATTAGCTTCGCCG-3´, 10 pmol primer 5´-TCATGTGGATCCA TGGAACGAGCCGAG-3´, 10 ng of cDNA, 1μl Taq polymerase, 10 μl of buffer (Eurogentec), 6 μl $MgCl_2$ (25 mM, Eurogentec) and 20 nmol of dNTPs in a total volume of 100 μl.

The mixture was denatured at 94°C for 5 min. 30 PCR cycles (60 sec at 95°C, 45 sec at 50°C, 45 sec at 72°C) were performed. After another 7 min incubation at 72°C, the mixture was cooled at 4 °C, and the DNA was electrophoresed on a 0.8 % agarose gel. The band at 750 bp was purified with a gel extraction kit (Qiagen). The DNA fragment was excised from the agarose gel with a scalpel. Three volumes of buffer QX1 (Qiagen) were added to 1 volume of the excised gel and incubated at 50°C for 10 min. One gel volume of isopropanol was added. To bind DNA, the sample was applied to a Qiaquick column and centrifuged for 1 min at 14000 rpm. The flow through was discarded. 0.75 ml buffer PE (Qiagen) were added to the column and centrifuged as before. The flow through was discarded and the column was centrifuged for an additional 1 min at 14000 rpm. The column was placed in a clean 1.5 ml Eppendorf tube. 50 μl of H_2O (bidestilled, sterile) were added to the column and it was centrifuged for 1 min at 14000 rpm. The flow through contained the purified DNA.

Second PCR (Two identical PCRs with 100 μl each were performed to obtain a

higher yield)

10 pmol primer CAATTTGAATTCATTAAAGAGGAGAAATTAACTATG-3´, 10 pmol 5´-TCATGTGGATCCATGGAACGAGCCGAG-3´, 1 µl of Taq polymerase (1 U), 10 µl of buffer (Taq-buffer, Eurogentec), 6 µl $MgCl_2$ (25 mM), 5 µl of purified PCR1 product and 20 nmol dNTPs in a total volume of 100 µl.
The mixture was denatured at 94°C for 5 min. 30 PCR cycles (60 sec at 94 °C, 45 sec at 50°C, 45 sec at 72 °C) were performed. After another 7 min incubation at 72°C, the mixture was cooled at 4°C, and the DNA was purified with a PCR purification kit (Qiagen).

5 volumes of buffer PB (Qiagen) were added to 1 volume of the PCR reaction, applied to a Qiaquick column and centrifuged for 1 min at 14000 rpm. The flow through was discarded. 0.75 ml buffer PE (Qiagen) were added to the column and centrifuged as before. Plasmid pNCO113 (Stüber et al., 1980) was isolated from 20 ml overnight culture of pNCO113 in *E. coli* XL-1 Blue using the plasmid isolation kit Nucleobond AX100 (Macherey & Nagel).

The PCR product and the plasmid pNCO113 were digested with the restriction enzymes EcoRI and BamHI, 20 µl OPA buffer (Pharmacia), 2 µl EcoRI (20 U, Pharmacia), 2 µl BamHI (20 U, Pharmacia), 2,5 µg PCR2 product resp. 5.0 µg pNCO113 in a total of 100 µl H_2O) at 37°C for 4 h and purified with a PCR purification kit. The digested PCR2 product and the plasmid pNCO113 were ligated together with T_4-ligase yielding plasmid pNCOribE(AT): 50 fmol of pNCO113, 100 fmol of PCR2 product, 4 µl of buffer (Gibco), and 1 µl of T_4-ligase (1 U, Gibco) in a total of 20 µl. The mixture was incubated overnight at 4°C, purified with a PCR purification kit and transformed into electrocompetent *E. coli* XL-1 Blue cells (Bullock et al., 1987) by electroporation.

Preparation of the electrocompetent cells: One liter of Luria-Bertani-medium was

inoculated with 10 ml of a fresh overnight culture. The cells were grown at 37 °C with vigorous shaking to an optical density of 0.5 to 0.7. The suspension was chilled on ice for 20 min and centrifuged in a cold rotor at 4000 g for 15 min at 4°C. The supernatant was removed and the pellet resuspended in 1 liter of ice-cold sterile 10 % glycerol. The cells were pelleted two times as described before and the pellet was resuspended the first time in 0.5 liter and the second time in 20 ml of ice-cold 10 % glycerol. The cells were again pelleted, and the pellet was resuspended to a final volume of 2 to 3 ml in ice-cold 10 % glycerol. The suspension was frozen in aliquots of 80 µl and stored in liquid nitrogen.

Electro-transformation using the Gene Pulser apparatus from Biorad: The electrocompetent cells were thawed at room temperature and placed on ice. 40 µl of the cell suspension were mixed with 1 µl of the ligation mixture and transferred into a sterile 0.2 cm cuvette (Biorad). The suspension was shaked to the bottom and the cuvette was placed into the chamber slide. The chamber slide was pushed into the chamber and a pulse was applied (2,50 kV, 25 µF, Pulse Controller 200 Ohm). The cuvette was removed from the chamber and the cells were suspended in 1 ml Soc medium (2 % casein hydrolysate, 0.5 % yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose). The suspension was incubated with shaking for 1 h at 37°C and plated on LB media supplemented with 150 mg ampicillin per liter. Plasmids from different clones were isolated (pNCOribE(AT)1-10).

The plasmids were isolated from 5 ml of fresh overnight culture using the mini plasmid isolation kit from Qiagen. The bacterial pellet was resuspended in 0.3 ml of 50 mM Tris hydrochloride, pH 8.0 containing 10 mM EDTA and 100 µg/ml RNase. 0.3 ml of 200 mM sodium hydroxide containing 1 % SDS were added, and the mixture was incubated for 5 min at room temperature. 0.3 ml of chilled 3.0 M sodium acetate, pH 5.5 were added, and the mixture was incubated on ice for 10 min. The mixture was centrifuged for 15 min at 14000 rpm in a

minifuge. The supernatant was removed and applied to a Qiagen-tip 20 which was previously equilibrated with 1 ml of 50 mM MOPS , pH 7.0, containing 750 mM NaCl, 15 % ethanol and 0.15 % Triton X-100. The Qiagen tip was washed 4 times with 1 ml of 50 mM MOPS, pH 7.0 containing 1000 mM NaCl and 1 % ethanol. The DNA was eluted with 0.8 ml of 50 mM Tris hydrochloride, pH 8.5 containing 1250 mM NaCl and 15 % ethanol. The DNA was precipitated with 0.7 volumes of isopropanol, centrifuged at 14000 rpm for 30 min and washed with 1 ml cold 70 % ethanol. The DNA sequence of the recombinant plasmids were determined by a automated dideoxynucleotide sequencing methode using an ABI Prism 377 DNA sequencer from Applied Biosystems Inc. with the ABI Prism Sequencing Analysis Software.

Example 2: Construction of an expression clone without the putative transit peptide sequence

The *ribE* gene with the exception of the first 216 bp coding for a putative transit peptide was amplified by PCR using plasmid pNCOribE(AT)1 (from *A. thaliana* ribE expression clone) as template. Plasmid pNCOribE(AT)1 was isolated as described. Amino acid 73 was mutated from an arginine to methionine and a recognition site for the restriction enzyme EcoRI preceding the start codon was introduced at the 5´ end with a modifying primer.

First PCR:
The reaction mixtures contained 10 pmol primer 5´-GGAGAAATTAACCATGCATGTTA-CGGGGTCTCTTATC-3´, 10 pmol primer 5´-TCATGTGGATCCATGGAACGAGCCGAG-3´, 10 ng of cDNA, 1 μl Taq polymerase (1 U), 10 μl of buffer (Eurogentec), 6 μl of $MgCl_2$ (25 mM, Eurogentec) and 20 nmol of dNTPs in a total volume of 100 μl.
The mixture was denatured at 94°C for 5 min. 30 PCR cycles (60 sec at 94°C, 45 sec at 50°C, 45 sec at 72°C) were performed. After another 7 min

incubation at 72°C, the mixture was cooled at 4°C, and the DNA was electrophoresed on a 0.8 % agarose gel. The band at 500 bp was purified with a gel extraction kit (Qiagen).

Second PCR (Two identical PCRs with 100 µl each were performed to obtain a higher yield)

10 pmol primer CAATTTGAATTCATTAAAGAGGAGAAATTAACTATG-3´, 10 pmol 5´-TCATGTGGATCCATGGAACGAGCCGAG-3´, 1 µl of Taq polymerase (1 U), 10 µl of buffer (Taq-buffer, Eurogentec), 6 µl of $MgCl_2$ (25 mM), 5 µl of purified PCR1 product and 20 nmol dNTPs in a total volume of 100 µl.
The mixture was denatured at 94°C for 5 min. 30 PCR cycles (60 sec at 94°C, 45 sec at 50°C, 45 sec at 72°C) were performed. After another 7 min incubation at 72°C, the mixture was cooled at 4°C, and the DNA was purified with a PCR purification kit (Qiagen).

The further steps were analogous to the construction of pNCOribE(AT) in Reference Example 1. The resulting plasmid encoding a ribE protein without the putative peptide sequence is designated pNCOribE(AT)-sig.

Example 3: Construction of an malE-ribE fusion clone without putative transit peptide sequence

The *ribE* gene with the exception of the first 216 bp was amplified by PCR using plasmid pNCOribE(AT) as template and was ligated in frame at the 3´-end of the *malE* gene.
First PCR:
The reaction mixtures contained 10 pmol primer 5´-ATAATAATAGCGGCCGCTATGCATGT-TACGGGGTCTCTTATC -3´, 10 pmol primer 5´-TCATGTGGATCCATGGAACGAGCCGAG-3´, 10 ng of cDNA, 1µl Taq

polymerase (1 U), 10 µl of buffer (Eurogentec), 6 µl $MgCl_2$ (25 mM, Eurogentec) and 20 nmol of dNTPs in a total volume of 100 µl.
The mixture was denatured at 94°C for 5 min. 30 PCR cycles (60 sec at 94° C, 45 sec at 50° C, 45 sec at 72°C) were performed. After another 7 min incubation at 72° C, the mixture was cooled at 4° C, and the DNA was electrophoresed on a 0.8 % agarose gel. The band at 500 bp was purified with a gel extraction kit (Qiagen).

Second PCR (Two identical PCRs with 100 µl each were performed to obtain a higher yield)
10 pmol primer CAATTTGAATTCATTAAAGAGGAGAAATTAACTATG-3´, 10 pmol 5´-TCATGTGGATCCATGGAACGAGCCGAG-3´, 1 µl of Taq polymerase (1 U), 10 µl of buffer (Taq-buffer, Eurogentec), 6 µl $MgCl_2$ (25 mM), 5 µl of purified PCR1 product and 20 nmol dNTPs in a total volume of 100 µl.
The mixture was denatured at 94° C for 5 min. 30 PCR cycles (60 sec at 94° C, 45 sec at 50° C, 45 sec at 72°C) were performed. After another 7 min incubation at 72° C, the mixture was cooled at 4° C, and the DNA was purified with a PCR purification kit (Qiagen).

Plasmid pNCOmalEribH (Fischer, 1997) was isolated from 20 ml of a overnight culture as described for plasmid pNCO113.
The PCR product and the plasmid pNCOmalEribH were digested with the restriction enzymes NotI and BamHI (10 µl BamHI buffer (NEB), 1 µl BSA (100x), 4 µl BamHI (20 U, NEB), 3 µl NotI (60 U, NEB), 2 µg PCR product resp. 5 µg pNCOmalEribH in a total volume of 100 µl) at 37° C for 3 h. The PCR product was purified with a PCR purification kit (Qiagen). The digested plasmid was electrophoresed on an agarose gel. The band at 3400 bp was purified with a gel extraction kit (Qiagen). Further steps were analogous to the construction of pNCOribE(AT) in Example 1. the plasmid encoding a malE-ribE fusion protein was named pNCOmalE-ribE(AT)-sig

Example 4: Preparation and purification of the ribE proteins with and without signal sequence

1 l Luria Bertani (LB) medium containing 160 mg ampicillin were inoculated with 40 ml overnight culture of *E. coli* strain XL-1 harboring plasmid pNCOribE(AT) resp. pNCOribE(AT)-sig. The culture was grown in shaking culture at 37°C. At an optical density (600 nm) of 0.6 the culture was induced with 2mM IPTG. The culture was grown for another 5 h. The cells were harvested by centrifugation for 20 min at 5000 rpm and 4°C. The cells were washed with 0.9 % NaCl solution, centrifugated as above and frozen at -20° C for storage. The cells were thawed in 20 ml 50 mM potassiumphosphate pH 7.0 containing 1 mM EDTA and 0.5 mM phenylmethylsulfonyl fluoride. The mixture was sonified 6 x 15 sec (Branson sonifier level 4). The suspension was centrifuged at 15000 rpm at 4°C for 20 min. The supernatant was applied to a 20 ml column of Sepharose Q (Pharmacia) previously equillibrated with 50 mM potassium phosphate pH 7.0 (buffer A). the column was washed with 60 ml buffer A and developed with a linear gradient of 200 ml buffer A containing 1 M NaCl. Fractions containing the ribE protein were identified by SDS electrophorese and concentrated with a Amicon cell (membran size, 30 kDa). Aliquots containing 5 mg protein were passed through a Superdex 200 (1.6 by 60 cm, Pharmacia) gel filtration column , which was developed with buffer A containing 100 mM NaCl. 38 mg ribE(AT) resp. 2 mg ribE(AT)-sig were obtained.

Example 5: Preparation and purification of the MBP-ribE fusion proteins

0.5 l Luria Bertani (LB) medium containing 75 mg ampicillin were inoculated with 40 ml overnight culture of *E. coli* strain XL-1 harboring plasmid pNCOmalEribE(AT). The culture was grown in shaking culture at 37°C. At an optical density (600 nm) of 0.5 the culture was induced with 1mM IPTG. The

culture was incubated with shaking for another 3 h. The cells were harvested by centrifugation for 20 min at 5000 rpm and 4°C. The cells were washed with 0.9 % NaCl solution, centrifuged as above and stored at -20°C. The cells were thawed in 10 ml 50 mM potassium phosphate, pH 7.0 containing 1 mM EDTA and 0.5 mM phenylmethylsulfonyl fluoride. The mixture was sonified 6 x 15 sec (Branson sonifier level 4). The suspension was centrifuged at 15000 rpm at 4°C for 20 min. The supernant was diluted 1:5 with buffer A (Example 4) and placed on a 2 ml column of amylose resin (New England Biolabs) previously equilibrated with 15 ml buffer A. The column was washed with 30 ml buffer A. The fusion protein was eluted from the column with 6 ml buffer A containing 10 mM maltose. 5 mg malE-ribE protein was obtained. The purity of the protein (56 kDa) was examined by SDS electrophorese.

Example 6: Screening for lumazine synthase activity

Preparation of 3,4-dihydroxy-2-butanon-4-phosphate:
A reaction mixture contained 100 mM potassium phosphate pH 7.5, 20 mM $MgCl_2$, 10 mM ribose 5-phosphate (Sigma) and 0.1 U pentose-phosphate isomerase (Sigma) and 3,4-dihydroxy-2-butanon 4-phosphate synthase (2000 U) in a total volume of 100 μl. The mixture was incubated at 37°C for 20 min.

Assay of lumazine synthase activity:
Assay mixture contained 100 mM potassium phosphate, pH 7.0, 20 mM EDTA, 1 mM 3,4-dihydroxy-2-butanone 4-phosphate, and 5 μl of the enzyme sample in a total volume of 50 μl. After a preincubation time of 2 min at 37°C the reaction was started by the addition of 1 μl of 5 mM 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione and the samples were incubated at 37°C for 5 min. The reaction was stopped by the addition of 50 ml trichloroacetic acid (15 %). 6,7-Dimethyl-8-ribityllumazine was determined by reverse-phase HPLC on a column of Nucleosil RP18. The eluent contained 30 mM formic acid and 20 %

methanol. The effluent was monitored fluorometrically (excitation, 408 nm; emission, 487 nm). One unit of enzyme activity catalyzes the formation of 1 nmol of 6,7-dimethyl-8-ribityllumazine per h at 37°C.

construct	specific activity (nmol h-1 mg-1)	
	cell extract	purified protein
E. coli XL-1 (host strain)	4	----
pNCOribE(AT)	48	8740
pNCOribE(AT)-sig	835	12000
pNCOmalE-ribE(AT)-sig	203	9130

Screening for inhibition of lumazine synthase activity

The screening for inhibition of lumazine synthase was done with the ribE gene product without the putative transit peptide sequence. The assays were performed with different inhibitor concentrations at constant concentrations of 5-amino-6-ribitylamino-2,4(1H,3H)pyrimidindione, and 3,4-dihydroxy-2-butanone 4-phosphate. The amount of biomimetically formed lumazine was substracted from the total amount of lumazine.

Screening for lumazine synthase activity in the presence of 5-nitroso-6-ribitylamino-2,4(1H,3H)pyrimidinedione (Winestock & Plaut, 1961) or 5-nitro-6-ribitylamino-2,4(1H,3H)pyrimidinedione (Cresswell & Wood, 1960)

test compound	5-nitro-6-ribitylamino-2,4(1H,3H)pyrimidinedione		5-nitroso-6-ribitylamino-2,4(1H,3H)pyrimidinedione	
concentration [μM]	lumazine formation per min [μM/min]	enzymatic activity [nmol/h mg]	lumazine formation per min [μM/min]	enzymatic activity [nmol/h mg]
0	26,41	9080 (100%)	27,09	9314 (100%)
5	26,41	9080 (100%)	26,21	9011 (97%)
10	25,73	8730 (96%)	25,14	8643 (93%)
100	20,00	6980 (77%)	23,40	8045 (86%)
1000	6,89	2070 (23%)	9,61	3304 (35%)
5000	3,01	980 (11%)	1,46	502 (5%)
10000	2,62	650 (7%)	1,07	368 (4%)
biomimetically formed lumazine	0,1		0,1	

Compound	IC_{50} [μM]
5-Nitro-6-ribitylamino-2,4(1H,3H)pyrimidinedione	310
5-Nitroso-6-ribitylamino-2,4(1H,3H)pyrimidinedione	560

500 μl potassium phosphate buffer (100 mM, pH 7.5) containing 1 mM EDTA and 10 μg enzyme, 100 μl inhibitor (final concentration, 0 - 10 mM), and 10 μl 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimdinedione (9 mM) were combined in a 1 ml cuvette. After a preincubation time of 10 min at 37°C, the reaction was started by the addition of 20 μl 3,4-dihydroxy-2-butanone 4-phosphate and incubated for another 5 min at 37°C. The reaction was monitored photometrically at 410 nm.

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Annex A

Nucleotide- and amino acid sequence of the lumazine synthase of *Arabidopsis thaliana*

```
ATGAAGTCATTAGCTTCGCCGCCGTGTCTCCGCCTGATACCGACGGCACACCGTCAGCTC
 M  K  S  L  A  S  P  P  C  L  R  L  I  P  T  A  H  R  Q  L

AATTCGCGTCAATCTTCCTCCGCCTGTTATATACACGGTGGCTCTTCTGTGAACAAATCC
 N  S  R  Q  S  S  S  A  C  Y  I  H  G  G  S  S  V  N  K  S

AATAATCTCTCATTCTCCTCATCCACATCCGGATTTGCGTCACCACTAGCTGTAGAGAAG
 N  N  L  S  F  S  S  S  T  S  G  F  A  S  P  L  A  V  E  K

GAATTACGCTCTTCATTCGTACAGACGGCTGCTGTTCGCCATGTTACGGGGTCTCTTATC
 E  L  R  S  S  F  V  Q  T  A  A  V  R  H  V  T  G  S  L  I

AGAGGCGAAGGTCTTAGATTCGCCATCGTGGTAGCTCGTTTCAATGAGGTTGTGACTAAG
 R  G  E  G  L  R  F  A  I  V  V  A  R  F  N  E  V  V  T  K

TTGCTTTTGGAAGGAGCGATTGAGACTTTCAAGAAGTATTCAGTCAGAGAAGAAGACATT
 L  L  L  E  G  A  I  E  T  F  K  K  Y  S  V  R  E  E  D  I

GAAGTTATTTGGGTTCCTGGCAGCTTTGAAATTGGTGTTGTTGCACAAAATCTTGGGAAA
 E  V  I  W  V  P  G  S  F  E  I  G  V  V  A  Q  N  L  G  K

TCGGGAAAATTTCATGCTGTTTTATGTATCGGCGCTGTGATAAGAGGAGATACCACACAT
 S  G  K  F  H  A  V  L  C  I  G  A  V  I  R  G  D  T  T  H

TATGATGCTGTTGCCAACTCTGCTGCGTCTGGAGTACTTTCTGCTAGCATAAATTCAGGC
 Y  D  A  V  A  N  S  A  A  S  G  V  L  S  A  S  I  N  S  G

GTTCCATGCATATTTGGTGTACTGACTTGCGAGGACATGGATCAGGCTCTGAATCGATCT
 V  P  C  I  F  G  V  L  T  C  E  D  M  D  Q  A  L  N  R  S

GGTGGCAAAGCCGGCAATAAGGGAGCTGAAACTGCTTTGACGGCGCTCGAAATGGCGTCG
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TTGTTTGAGCACCACCTGAAATAGCTCGGCTCGTTCCAT
 L  F  E  H  H  L  K
```

Claims

1. A method for screening for the presence or absence of inhibition of 6,7-dimethyl-8-ribityllumazine synthase activity comprising the following steps:
   (a) preparing a first aqueous mixture containing a protein having a plant-type 6,7-dimethyl-8-ribityllumazine synthase sequence, 5-amino-6-ribitylamino-2,4(1H,3H)pyrimidine-dione and 3,4-dihydroxy-2-butanone 4-phosphate;
   (b) reacting said first mixture during a predetermined period of time at a predetermined temperature and subsequently detecting the level of 6,7-dimethyl-8-ribityllumazine;
   (c) preparing a second aqueous mixture by including in said first mixture a predetermined amount of a chemical test sample;
   (d) reacting said second mixture during the predetermined period of time at the predetermined temperature and subsequently detecting the level of 6,7-dimethyl-8-ribityllumazine;
   (e) determining the presence of inhibition of 6,7-dimethyl-8-ribityllumazine synthase by observation of whether the level detected in step (d) is lower than the level detected in step (b).

2. A method for screening for the presence or absence of resistance to inhibition of 6,7-dimethyl-8-ribityllumazine synthase activity comprising the following steps:
   (a) preparing a first aqueous mixture containing a protein having a mutated plant-type 6,7-dimethyl-8-ribityllumazine synthase sequence, 5-amino-6-ribityl amino-2,4(1H,3H)pyrimidione and 3,4-dihydroxy-2-butanone 4-phosphate;
   (b) reacting said first mixture during a predetermined period of time at

a predetermined temperature and subsequently detecting the level of 6,7-dimethyl-8-ribityllumazine;

(c) preparing a second aqueous mixture by including in said first mixture a predetermined amount of a specific inhibitor for 6,7-dimethyl-8-ribityllumazine synthase activity;

(d) reacting said second mixture during the predetermined period of time at the predetermined temperature and subsequently detecting the level of 6,7-dimethyl-8-ribityllumazine;

(e) determining the presence of resistance to inhibition of 6,7-dimethyl-8-ribityllumazine synthase by observation of whether the level detected in step (d) is similar to the level detected in step (b).

3. The method according to claims 1 or 2, wherein said aqueous mixture has a pH in the range of 5.5 to 9.

4. The method according to claims 1 or 2, wherein a premixture is prepared which lacks one essential ingredient and the reaction is started by adding said ingredient.

5. The method according to claims 1 or 2, characterized in that the reaction is terminated by adding an acid or a solvent or a surfactant, preferably trichloroacetic acid or acetone or sodium dodecylsulfate.

6. The method according to one of the claims 1 to 5, characterized in that the level of 6,7-dimethyl-8-ribityllumazine is detected photometrically or fluorometrically.

7. The method according to claim 6, wherein the detection is effected in an HPLC fraction.

8. The method according to one of claims 1 to 6, characterized in that the detection is effected by incubation with riboflavin synthase and detection of riboflavin.

9. The method according to one of claims 1 or 2, characterized in that a mixture containing 3,4-dihydroxy-2-butanone 4-phosphate is prepared by incubating an aqueous mixture containing ribulose 5-phosphate with 3,4-dihydroxy-2-butanone 4-phosphate synthase.

10. The method according to one of claims 1 or 2, characterized in that a mixture containing 3,4-dihydroxy-2-butanone 4-phosphate is prepared by incubating an aqueous mixture containing ribose 5-phosphate with pentose-phosphate isomerase and 3,4-dihydroxy-2-butanone 4-phosphate synthase.

11. An isolated protein having a plant-type 6,7-dimethyl-8-ribityllumazine synthase sequence and existing in a form functional for 6,7-dimethyl-8-ribityllumazine synthase activity.

12. An isolated DNA coding exclusively for
    (a) a protein comprising a plant-type 6,7-dimethyl-8-ribityllumazine synthase sequence; and
    (b) optionally at least one additional enzyme of the flavin biosynthetic pathways.

13. A method of inhibiting an enzyme with 6,7-dimethyl-8-ribityllumazine synthase activity of or in a plant by treatment with a compound selected from the group of chemical compounds that exhibit inhibition in the screening method of claim 1.

14. A chemical compound exhibiting inhibition of a plant 6,7-dimethyl-8-ribityllumazine synthase activity in the method of claim 1.

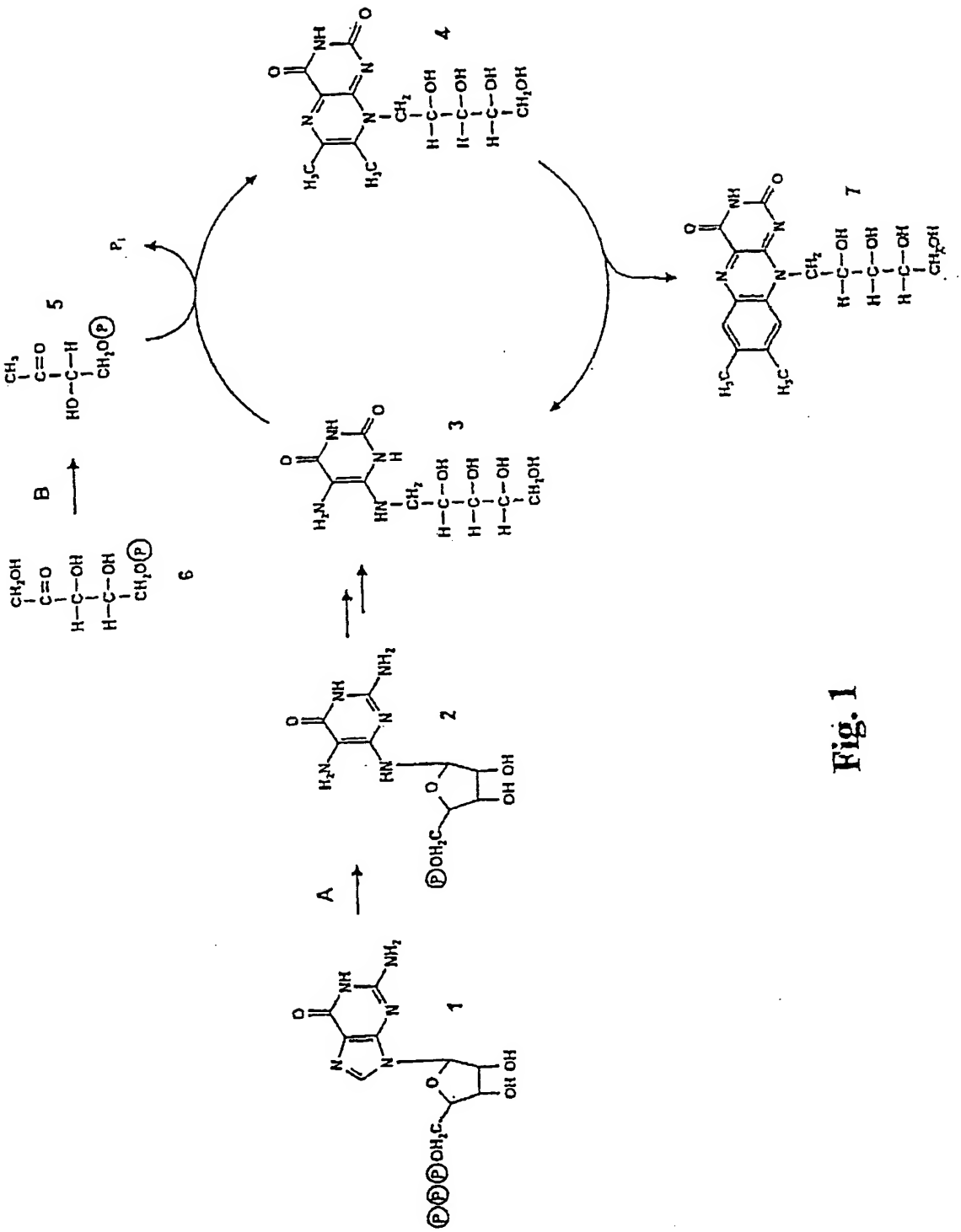

Fig. 1

```
1. MKSLASPPCLRLIPTAHRQLNSRQSSSACYIHGGSSVNKSNNLSFSSST
2.
3.

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2.                            MNIIEANVATPDARVAITIARFNNFI
3.                            MNIIQGNLVGTGLKIGIVVGRFNDFI

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2. NDSLLEGAIDALKRIGQVKDENITVVWVPGAYELPLAAGALAKTGKYDA
3. TSKLLSGAEDALLRHG-VDTNDIDVAWVPGAFEIPFAAKKMAETKKYDA

1. VLCIGAVIRGDTTHYDAVANSAASGVLSASINSGVPCIFGVLTCEDMDQ
2. VIALGTVIRGGTAHFEYVAGGASNGLAHVAQDSEIPVAFGVLTTESIEQ
3. IITLGTVIRGATTHYDYVCNEAAKGIAQAANTTGVPVIFGIVTTENIEQ

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2. AIERAGTKAGNKGAEAALTALEMINVLKAIKA    E.coli
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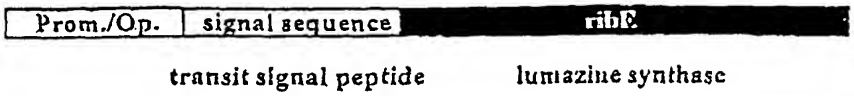
Fig. 2

(A) pNCO-sig-ribE



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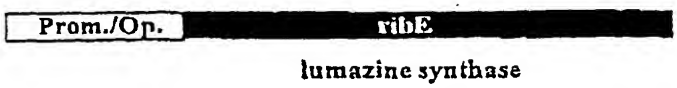

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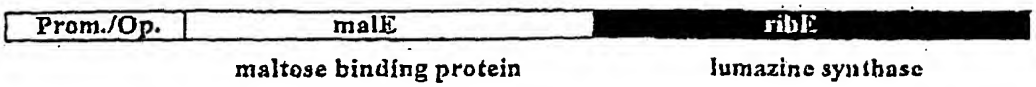

Fig. 3

SEQUENCE LISTING

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<120> Method for screening for inhibitors of the biosynthesis of riboflavin

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His Arg Gln Leu Asn Ser Arg Gln Ser Ser Ser Ala Cys Tyr Ile His
20 25 30

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         35                  40                  45

aca tcc gga ttt gcg tca cca cta gct gta gag aag gaa tta cgc tct   192
Thr Ser Gly Phe Ala Ser Pro Leu Ala Val Glu Lys Glu Leu Arg Ser
     50                  55                  60

tca ttc gta cag acg gct gct gtt cgc cat gtt acg ggg tct ctt atc   240
Ser Phe Val Gln Thr Ala Ala Val Arg His Val Thr Gly Ser Leu Ile
 65                  70                  75                  80

aga ggc gaa ggt ctt aga ttc gcc atc gtg gta gct cgt ttc aat gag   288
Arg Gly Glu Gly Leu Arg Phe Ala Ile Val Val Ala Arg Phe Asn Glu
                 85                  90                  95

gtt gtg act aag ttg ctt ttg gaa gga gcg att gag act ttc aag aag   336
Val Val Thr Lys Leu Leu Leu Glu Gly Ala Ile Glu Thr Phe Lys Lys
            100                 105                 110

tat tca gtc aga gaa gaa gac att gaa gtt att tgg gtt cct ggc agc   384
Tyr Ser Val Arg Glu Glu Asp Ile Glu Val Ile Trp Val Pro Gly Ser
        115                 120                 125

ttt gaa att ggt gtt gtt gca caa aat ctt ggg aaa tcg gga aaa ttt   432
Phe Glu Ile Gly Val Val Ala Gln Asn Leu Gly Lys Ser Gly Lys Phe
    130                 135                 140

cat gct gtt tta tgt atc ggc gct gtg ata aga gga gat acc aca cat   480
His Ala Val Leu Cys Ile Gly Ala Val Ile Arg Gly Asp Thr Thr His
145                 150                 155                 160

tat gat gct gtt gcc aac tct gct gcg tct gga gta ctt tct gct agc   528
Tyr Asp Ala Val Ala Asn Ser Ala Ala Ser Gly Val Leu Ser Ala Ser
                165                 170                 175

ata aat tca ggc gtt cca tgc ata ttt ggt gta ctg act tgc gag gac   576
Ile Asn Ser Gly Val Pro Cys Ile Phe Gly Val Leu Thr Cys Glu Asp
            180                 185                 190

atg gat cag gct ctg aat cga tct ggt ggc aaa gcc ggc aat aag gga   624
Met Asp Gln Ala Leu Asn Arg Ser Gly Gly Lys Ala Gly Asn Lys Gly
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gct gaa act gct ttg acg gcg ctc gaa atg gcg tcg ttg ttt gag cac   672
Ala Glu Thr Ala Leu Thr Ala Leu Glu Met Ala Ser Leu Phe Glu His
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699

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35 40 45

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Val Val Thr Lys Leu Leu Leu Glu Gly Ala Ile Glu Thr Phe Lys Lys
100 105 110

Tyr Ser Val Arg Glu Glu Asp Ile Glu Val Ile Trp Val Pro Gly Ser
115 120 125

Phe Glu Ile Gly Val Val Ala Gln Asn Leu Gly Lys Ser Gly Lys Phe
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20 25 30

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100 105 110

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35 40 45

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50 55 60

Thr Gly Val Pro Val Ile Phe Gly Ile Val Thr Thr Glu Asn Ile Glu
65 70 75 80

Gln Ala Ile Glu Arg Ala Gly Thr Lys Ala Gly Asn Lys Gly Val Asp
85 90 95

Cys Ala Val Ser Ala Ile Glu Met Ala Asn Leu Asn Arg Ser Phe Glu
100 105 110

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

HARTZ, Nikolai
Wächtershäuser & Hartz
Tal 29
80331 München
ALLEMAGNE

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	04.12.2001
Applicant's or agent's file reference PCT-11222	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP00/07796	10/08/2000	03/09/1999

Applicant
BACHER, Adelbert et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Digiusto, M Tel.+49 89 2399-8162

PATENT COOPERATION TREATY

PCT

12

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
PCT-11222		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP00/07796	10/08/2000	03/09/1999
International Patent Classification (IPC) or national classification and IPC C12Q1/48		
Applicant BACHER, Adelbert et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 8 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☒ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
27/03/2001	04.12.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Barz, W Telephone No. +49 89 2399 7320

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP00/07796

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-20 as originally filed

Claims, No.:

1-14 as originally filed

Drawings, sheets:

1/3-3/3 as originally filed

Sequence listing part of the description, pages:

1-8, as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☒ contained in the international application in written form.
- ☒ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

   ☐ the entire international application.

   ☒ claims Nos. 13-14.

because:

   ☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):

   ☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

   ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

   ☒ no international search report has been established for the said claims Nos. 13-14.

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

   ☐ the written form has not been furnished or does not comply with the standard.

   ☐ the computer readable form has not been furnished or does not comply with the standard.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	4, 8-9
	No:	Claims	1-3, 5-7, 10-12
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-12
Industrial applicability (IA)	Yes:	Claims	1-12
	No:	Claims	

2. Citations and explanations
see separate sheet

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

ITEM III:

Due to the lack of disclosure (Article 5 PCT) as well as support and clarity (Article 6 PCT), the International Search was not performed over the whole scope of **claims 13-14** which are defined by a desirable characteristic (see Box I.2 of the International Search Report). Consequently, said claims will not be the subject of International Preliminary Examination (Rule 66.1(e) PCT).

ITEM V:

Reference is made to the following documents:

D1: WO 99 38986 A (NOVARTIS AG), 5 August 1999;
D2: JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 274, no. 31, 30 July 1999, pages 22114-22121, (DOUGLAS D.B. et al.), ISSN: 0021-9258;
D3: DATABASE EMBL - TREMBL [Online] ID/ACC: 080575, 1 November 1998, (ROUNSLEY, S D. et al.).

1. NOVELTY

Claims 1-3, 5-7, and 10-12 do not meet the requirements of Article 33(2) PCT for the following reasons:

1.1 A screening method comprising all features of present **claim 1** is disclosed in document D1 (abstract; page 11, paragraphs 2-3; examples 9-10; claim 50). Therefore, the subject-matter of claim 1 is not novel in the sense of Article 33(2) PCT.

1.2 Similarly, the screening method of present **claim 2** is also known from D1 (abstract; page 1, lines 9-11; page 7, lines 16-18; page 10, lines 1-9; page 12, 3rd paragraph; pages 22-30; claim 21). Consequently, novelty of said claim also cannot be acknowledged.

1.3 The dependent **claims 3, 5-7, and 10** do not contain any features which, in combination with the features of any claim to which they refer, meet the requirements of the PCT with respect to novelty, because the combination of features of said claims is also disclosed in D1 (see examples 6 and 9 as well as claim 52).

1.4 The subject-matter of present **claim 11** is also known from the prior art, because documents D1 (abstract; example 8; claim 44), D2 (abstract; page 22117, left column; figures 4-6), and D3 (abstract) disclose isolated plant proteins having the sequence and activity of lumazine synthase.

1.5 Similarly, the isolated DNA of present **claim 12** is disclosed in D1 (abstract; claims 1-4), D2 (abstract; paragraph bridging pages 22116-22117; figures 2), and D3 (abstract).

1.6 The remaining **claims 4 and 8-9** appear to be novel, because none of the available prior art documents discloses methods having the same combination of features as these claims.

2. INVENTIVE STEP

However, **claims 4 and 8-9** do not appear to be inventive in the sense of Article 33(3) PCT for the following reasons:

2.1 Compared to the method of D1 (abstract; examples 9-10; claim 50), the subject-matter of **claim 4** differs only in that a premixture lacking one essential ingredient is prepared. However, said modification is a standard procedure followed by persons skilled in the art, especially as the advantages thus achieved can readily be foreseen. Consequently, the subject-matter of claim 4 appears to lack an inventive step (Article 33(3) PCT).

2.2 Compared to D1, the subject-matter of **claim 8** differs only in that riboflavin is detected (instead of lumazine). Since, however, is it well known to the skilled person that lumazine is the immediate precursor of riboflavin in the riboflavin biosynthesis pathway, it would be obvious to the skilled person to detect riboflavin

(instead of lumazine) in order to detect lumazine synthase activity. Therefore, riboflavin detection can be considered an equivalent to lumazine detection and can thus be interchanged with that feature where circumstances make it desirable.

2.3 **Claims 9** also does not appear to involve an inventive step (Article 33(3) PCT), because the preparation of 3,4-dihydroxy-2-butanone-4-phosphate from ribulose-5-phosphate (instead of ribose-5-phosphate used in D1) is a well-known equivalent which can be interchanged with that feature where circumstances make it desirable.

3. INDUSTRIAL APPLICABILITY

The subject-matter of **claims 1-12** appears to be industrially applicable in the sense of Article 33(4) PCT.

ITEM VI:

Certain published documents (Rule 70.10)

Application No Patent No	Publication date *(day/month/year)*	Filing date *(day/month/year)*	Priority date *(valid claim)* *(day/month/year)*
WO 00 44727 A	03.08.2000	27.01.2000	30.01.1999
WO 00 40744 A	13.07.2000	14.12.1999	15.12.1998
EP-A-1 010 760	21.06.2000	11.12.1998	---

The claim to priority of the present application appears to be valid. Therefore, the above patent documents which were published after the priority date, but before the filing date of the present application (listed as "P,X" documents in the International Search Report) do not belong to the state of the art (Rule 64.1 PCT) for the present PCT application. However, said patent documents may become relevant prior art in the Regional phase of the present application.

ITEM VII:

Contrary to the requirements of Rule 5.1(a)(ii) PCT, the relevant background art disclosed in the documents D1-D3 is not mentioned in the description, nor are these documents identified therein.

ITEM VIII:

1. The term "plant-type" used in **claims 1-2 and 11-12** is unclear (Article 6 PCT), because its meaning is not apparent from the wording of the claim alone (PCT Guidelines III-4.2). Furthermore, since even the definition of said term in the description (page 7, 2nd paragraph) is not clear, it would not appear clear to the skilled person to which technical features said term is referring.

2. **Claims 3, 5, and 8-9** do not fulfill the requirements of Article 6 PCT, because there features do not appear to be supported by the description.

3. The term "premixture" used in **claim 4** is not clear in the sense of Article 6 PCT, because the contents of said premixture is not defined. Therefore, it is not apparent whether the "first aqueous mixture" or "second aqueous mixture" (or both) are premixed.

Translation

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 662220	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/JP00/07857	International filing date (*day/month/year*) 09 November 2000 (09.11.00)	Priority date (*day/month/year*) 10 November 1999 (10.11.99)
International Patent Classification (IPC) or national classification and IPC H04N 5/92		
Applicant MATSUSHITA ELECTRIC INDUSTRIAL CO., LTD.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 7 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
07 June 2001 (07.06.01)	04 March 2002 (04.03.2002)
Name and mailing address of the IPEA/JP Facsimile No.	Authorized officer Telephone No.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

ernational application No.
PCT/JP00/07857

I. Basis of the report

1. With regard to the **elements** of the international application:*

☐ the international application as originally filed

☒ the description:

pages 1,4-11, as originally filed
pages 3, filed with the demand
pages 2,3/1, filed with the letter of 07 September 2001 (07.09.2001)

☒ the claims:

pages 2,10,11, as originally filed
pages _____, as amended (together with any statement under Article 19
pages _____, filed with the demand
pages 1,4-9, filed with the letter of 07 September 2001 (07.09.2001)

☒ the drawings:

pages 1-6, as originally filed
pages _____, filed with the demand
pages _____, filed with the letter of _____

☐ the sequence listing part of the description:

pages _____, as originally filed
pages _____, filed with the demand
pages _____, filed with the letter of _____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

☐ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

☐ the language of publication of the international application (under Rule 48.3(b)).

☐ the language of the translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

☐ contained in the international application in written form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. ☒ The amendments have resulted in the cancellation of:

☒ the description, pages 2/1
☒ the claims, Nos. 3
☐ the drawings, sheets/fig _____

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

* *Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rule 70.16 and 70.17).*

** *Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

V. **Reasoned statement under Article 35(2) with regard to novelty, inventive step r industrial applicability; citations and explanations supporting such statement**

1. Statement

Novelty (N)	Claims	1-2, 4-11	YES
	Claims		NO
Inventive step (IS)	Claims	1-2, 4-11	YES
	Claims		NO
Industrial applicability (IA)	Claims	1-2, 4-11	YES
	Claims		NO

2. Citations and explanations

Claims 1, 2 and 4 to 11

Document 1: JP, 7-284056, A (Hitachi, Ltd.), October 27, 1995

Document 2: JP, 6-334961, A (Canon Inc.), December 2, 1994

Document 3: JP, 5-30462, A (Sony Corp.), February 5, 1993, paragraph [0020]; Fig. 4

Document 4: JP, 3-177176, A (Hitachi, Ltd.), August 1, 1991

Document 5: JP, 7-135635, A (Hitachi, Ltd.), May 23, 1995

Document 6: JP, 7-230666, A (Mitsubishi Electric Corp.), August 29, 1995

Documents 1 to 7 are documents that reflect the general state of the art in this technical field and disclose magnetic medium recording/playback devices that are capable of processing various types of image signals by means of format conversion. However, the feature of providing a delay circuit in order to synchronise and output a predetermined image signal and an image signal in which the aforementioned image signal has undergone format conversion, the feature of outputting format information to the output terminal of the image signal, the feature of outputting a warning when the designated format is different, and the feature of outputting a fixed pattern

when an abnormality occurs in the processing are neither disclosed nor suggested in any of the documents cited in the international search report.

TENT COOPERATION TRE/

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United Stat s Patent and Trademark
Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 29 May 2001 (29.05.01)	
International application No. PCT/EP00/07796	**Applicant's or agent's file reference** PCT-11222
International filing date (day/month/year) 10 August 2000 (10.08.00)	**Priority date** (day/month/year) 03 September 1999 (03.09.99)
Applicant BACHER, Adelbert et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

27 March 2001 (27.03.01)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

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